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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	6
Key Research Accomplishments.....	23
Reportable Outcomes.....	24
Conclusions.....	25
References.....	28
Appendices.....	33

INTRODUCTION

The HER-2 (*neu/erbB-2*) gene encodes a 185 kDa protein tyrosine kinase that is highly homologous to the epidermal growth factor (EGF) receptor (EGFR/HER-1/*erbB-1*), HER-3 (*erbB-3*), and HER-4 (*erbB-4*) (1-3), which together, comprise the type 1 family of receptor tyrosine kinases (4, 5). These receptors differ in their ligand specificities (4), and while HER-1 binds several ligands closely related to EGF, HER-3 and HER-4 are the receptors for a number of different isoforms of *neu* differentiation factor/hereregulin (HRG) (6-8). While no direct ligand for HER-2 has yet been cloned, it is now clear that HER-2 is capable of heterodimerization with HER-1 (9, 10), HER-3 (11, 12), or HER-4 (8), and these HER-2-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). HER-2 is amplified in 28% of primary breast carcinomas *in vivo* (13), and another 10% overexpress HER-2 without amplification of the gene (14-16). HER-2 gene amplification, concordant with high-level overexpression, is associated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17-19). Experimentally elevated HER-2 gene expression in various cell lines induces tumorigenesis in nude mice (24-27), and the potent oncogenic potential of HER-2 is generally thought to be due to its ability to constitutively activate key signaling pathways that are involved in the regulation of cell growth (28). Although HER-2 was originally discovered as the *neu* transmembrane-mutated form of the gene in rat neuroblastoma cells (29), the HER-2 gene found in human breast cancer cells has never shown such mutations (30). The level of tyrosine-phosphorylated HER-2 in primary breast cancer *in vivo* always shows a direct correspondence with HER-2 overexpression (31), and while the wild-type HER-2 protein possess constitutive tyrosine kinase activity when overexpressed in cell lines in the absence of any identifiable ligand (24-27, 32, 33), the HER-2 tyrosine kinase domain is also constitutively active in EGFR-HER-2 chimeric receptors in the absence of EGF (32, 33). Therefore, the overexpression of wild-type HER-2 alone is sufficient to constitutively activate its tyrosine kinase function. Additionally, heterodimeric interactions are now known to occur between the different HER proteins, including HER-1 and HER-2 (9, 10), HER-2 and HER-3 (11, 12), HER-2 and HER-4 (8), and HER-1 and HER-3 (34, 35). Our own work and that of others has now shown that heterodimer interactions between HER-2 and HER-3 are constitutively active in breast cancer cells with HER-2 gene amplification (20-23), and co-transfection of HER-3 with HER-2 greatly augments the transforming capability of HER-2 in genetically engineered cell lines (21). Therefore, we are particularly interested in how the cooperative effects of HER-2/HER-3 heterodimers activate key mitogenic signaling pathways which facilitate cell growth. Our own work and that of others has also now shown that the constitutive activation of HER-2/HER-3 in breast cancer cells is associated with the constitutive activation of phosphatidylinositol (PI) 3-kinase and mitogen-activated protein (MAP)-kinase (20-22). One strategy that has been used successfully to block the function of other receptor tyrosine kinases employs dominant negative expression vectors in which a region coding for the cytoplasmic domain of the receptor is almost completely removed. While the truncated receptor still contains the extracellular and transmembrane domains and can dimerize within the cell membrane, it lacks tyrosine kinase activity and inhibits the signal transduction docking function of both mutant/mutant homodimers and mutant/wild-type heterodimers (Fig. 1A). This strategy has been used effectively to block EGFR (36), platelet-derived growth factor receptor (37), and fibroblast growth factor receptor (38) in biochemistry studies. Recently, a dominant negative HER-2 vector was also used successfully to block HER-2 function in normal mouse

development (40). The use of such dominant negative HER-2 vectors has not yet been reported to block HER-2 in cancer cells with HER-2 gene amplification, and this is likely due to the stoichiometric problems associated with the inability to generate mutant/wild-type levels high enough for effective inhibition in human breast cancer cells with HER-2 gene amplification. However, the fact that HER-3 is not highly overexpressed in these cells, and that activated HER/HER-3 has a particularly high-affinity interaction (42-44), suggests that dominant negative HER-3 may be especially effective in blocking HER-2/HER-3 function. Therefore, we have introduced a dominant negative form of HER-3 into cells in an attempt to block the activation of HER-2/HER-3 (45; Fig. 1B).

BODY

As discussed in detail in the original grant proposal, we routinely use the H16N-2 normal breast epithelial cells and the 21MT-1 breast carcinoma cells for our studies because they were derived from the same patient and can be grown under precisely defined serum-free conditions in culture. This well defined system was used to study growth factor-independent (i.e. autonomous) proliferation and responses to exogenous growth factors in a manner that was not yet possible for other cell lines derived in medium containing high levels of serum and that are routinely propagated under undefined conditions in culture. Therefore, this serum-free system was well suited to study receptor activation and signaling in a manner which allowed us to distinguish constitutive from externally mediated growth factor responses in culture (22, 41, 45). H16N-2 and 21MT-1 cells were previously infected with the pCMV control vector or pCMV-dn3, selected on G418 and were used to determine the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture (45). Interestingly, dominant negative HER-3 had no apparent effect on the insulin/EGF-induced proliferation of either H16N-2 or 21MT-1 cells that had been infected with pCMV-dn3 (45; Fig. 2). This suggested that dominant negative HER-3 preferentially inhibited HER-2/HER-3-mediated growth responses over those mediated by exogenous EGF in H16N-2 and 21MT-1 cells that express EGFR and are highly responsive to EGF-induced proliferation. Thus the effects of pCMV-dn3 showed selectivity by preferentially inhibiting growth responses involving HER-2/HER-3, such as those that are constitutively activated by HER-2 or that are induced by HRG. This also indicated that HER-1/HER-3 is not as effectively inhibited by dominant negative HER-3 as is HER-2/HER-3 at a given level of dominant negative HER-3 gene expression or that the interaction between EGFR and HER-3 is not required for EGF-induced proliferation. Apparently, it was this preferential inhibition of HER-2/HER-3 that then allowed us to use a constitutive promoter to express dominant negative HER-3 in cells that were still able to proliferate in response to EGF. While EGFR and HER-3 do interact in these and other cell lines (46), clearly the EGFR/HER-3 heterodimer interaction is weak compared to the HER-2/HER-3 heterodimer interaction (42-44). To further investigate the preferential effects of dominant negative HER-3 on HER-2/HER-3-mediated growth responses in the 2MT-1 cells, we also compared the levels of receptor activation in cells stimulated with either HRG or EGF (Fig. 3). While dominant negative HER-3 inhibited the constitutive tyrosine phosphorylation of HER-2/HER-3 as well as that seen with HRG stimulation, the EGF-induced tyrosine phosphorylation of EGFR-containing dimers was apparently not effected by dominant negative HER-3 in the 21MT-1 cells. These results are consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers at a given level of dominant negative HER-3 gene expression.

In vivo studies were then performed using the 21MT-1-derived cell lines for injections into immunodeficient mice as described in the original grant proposal. However, the 21MT-1 cells were not tumorigenic in our studies and thus would not allow us to test the effects of dominant negative HER-3 *in vivo*. This is apparently a common problem for a significant number of malignant and metastatic breast carcinoma cell lines (47), and earlier studies also suggested some difficulty in using 21MT-1 cells for tumor studies at later passages (48). Additional experiments were performed during the grant period comparing injections of 21MT-1 cells in both Nu/Nu and Nu/Nu CD-1 strains of nude mice as well as in scid mice that lack both T-cell and B-cell function. We were not able to detect any tumors in mice injected with up to

10^7 21MT-1 cells per site in any of the strains of immunodeficient mouse yet tested. Interestingly, we also noticed on further review of the literature that previous studies employed fibroblasts genetically engineered to overexpress HER-2 for their *in vivo* experiments rather than spontaneously derived breast carcinoma cell lines with HER-2 gene amplification. We have also now done experiments using the BT-474, MDA-MB-453 and SK-BR-3 breast carcinoma cells for injection into immunodeficient mice. BT-474, MDA-MB-453 and SK-BR-3 cells have all been reported to contain HER-2 gene amplification and activation of HER-2/HER-3, so we had proposed to use these cell lines as well. After obtaining the cell lines from the ATCC, experiments were conducted to measure the level of their HER-2/HER-3 expression and receptor activation in culture as well as their tumorigenicity *in vivo*. None of these cell lines has yet formed detectable tumors in either Nu/Nu or Nu/Nu CD-1 strains of nude mice injected with 10^7 cells per injection. However, BT-474 cells were found to consistently form measurable tumors in scid mice within a few weeks after injection. In two groups of 8 injections each, detectable BT-474 tumors were dissected out and measured after 2 months from the time of injection of 10^7 cells per site in scid mice. The mean tumor diameter for each group of 8 injections was 4.1 ± 1.7 mm and 6.0 ± 2.1 mm, respectively. Experiments injecting MDA-MB-453 or SK-BR-3 cells in scid mice have also shown some very small tumors, but these tumors were detected only after autopsy more than 5 months after the time of injection.

Analysis of HER-2 and HER-3 expression was also carried out using the BT-474, MDA-MB-453 and SK-BR-3 cell lines. All 3 of these cell lines showed very high levels of HER-2, but was highest in the BT-474 cells (data not shown). HER-3 was expressed by all 3 cell lines, and was moderately elevated in the BT-474 and MDA-MB-453 cells relative to the SK-BR-3 cells (data not shown). In addition, the constitutive activation of HER-2 and HER-3 was also measured in the BT-474, MDA-MB-453 and SK-BR-3 cells (Fig. 4). The cells were placed in serum-free medium for 48 hours prior to protein extractions so that the levels of tyrosine-phosphorylated receptors represented the endogenous *constitutive* level of receptor activation in these cells. Immunoprecipitation/Western blot measurement of tyrosine-phosphorylated HER-2 and HER-3 showed that both HER-2 and HER-3 were most highly tyrosine-phosphorylated in the BT-474 cells. SK-BR-3 cells showed moderately high levels of HER-2 tyrosine-phosphorylation, but lower levels of HER-3 tyrosine-phosphorylation. MDA-MB-453 cells showed the lowest levels of HER-2 and HER-3 tyrosine-phosphorylation (Fig. 4). Western blotting of PI 3-kinase and SHC proteins in anti-phosphotyrosine immunoprecipitates also showed that BT-474 cells had much higher levels of p85^{PI 3 kinase}, p46^{SHC} and p52^{SHC} recruited by activated HER-2/HER-3 than that seen in the MDA-MB-453 cells and SK-BR-3 cells (data not shown).

We infected BT-474, MDA-MB-453 and SK-BR-3 cells with pCMV and pCMV-dn3 as outlined in the original grant proposal. Titrations were initially performed to determine the concentrations of G418 necessary to kill BT-474, MDA-MB-453 and SK-BR-3 cells within 2-3 weeks after addition of the antibiotic. These concentrations (i.e. 500 ug/ml for SK-BR-3 cells, 600 ug/ml for MDA-MB-453 cells and 700 ug/ml for BT-474 cells) were then used to select these cell lines on G418 after being infected with pCMV or pCMV-dn3. Infections of all the cell lines were first carried out using ψ CRIP-derived packaging cell lines previously transfected with pCMV or pCMV-dn3 and selected on G418. However, no colonies grew out for BT-474, MDA-MB-453 and SK-BR-3 cells infected with conditioned medium prepared from ψ CRIP-derived packaging cell lines. These ψ CRIP packaging cells are not thought to produce very high viral titers compared to other packaging cell lines freshly selected for packaging function. Therefore,

function. Therefore, we then transfected the PA-317 packaging cell line (ATCC) with pCMV or pCMV-dn3 and selected them on 800 ug/ml G418 for a month to derive new packaging cell lines which were then used for infecting BT-474, MDA-MB-453 and SK-BR-3 cells. By using the PA-317-derived packaging cell lines, we then successfully generated viral titers sufficient for good colony formation of BT-474, MDA-MB-453 and SK-BR-3 cells infected with the pCMV control vector. Interestingly, we also saw a much lower number of colonies growing out for the cells infected with pCMV-dn3 under identical conditions in culture. Earlier observations were then extended with additional experiments done in triplicate where the colonies were counted after infection and selection on G418 for a month in culture (Fig. 5). Furthermore, we also noticed that many of the colonies that started to grow out in plates infected with pCMV-dn3 were either very small (Fig. 6B) or did not continue to grow, instead showing gradual morphological deterioration and eventual cell death (data not shown). This suggested that the growth of many of the cells infected with pCMV-dn3 may be inhibited by the expression of dominant negative HER-3 during their selection on G418.

In order to confirm the expression of dominant negative HER-3 in BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV-dn3, we performed anti-HER-3 immunocytochemistry on cells infected with pCMV or pCMV-dn3 after being selected on G418 and passaged as pooled colonies (i.e. mass selected) in culture. The H105 monoclonal anti-HER-3 antibody (Neomarkers) is known to be highly specific for HER-3 and binds to an epitope within the extracellular domain of HER-3. While this antibody does not work for Western blotting, it works well for immunocytochemistry (45). While all of the cell lines used in these studies express wild-type HER-3, the levels are relatively low when detected with HRP/DAB staining in cells infected with pCMV compared to cells infected with pCMV-dn3 (45). Therefore, the high levels of HER-3 measured in cells infected with pCMV-dn3 confirmed the ectopic expression of dominant negative HER-3. However, we had also noticed significant heterogeneity in the levels of HER-3 staining in cell populations infected with pCMV-dn3 (Fig. 5 in 2001 annual report). Such heterogeneity in dominant negative HER-3 expression in these cells also suggested the possibility that there may be a preferential selection of cells expressing lower levels of dominant negative HER-3 if it inhibits the growth of these cells during the initial selection on G418. These mass selected BT-474, MDA-MB-453 and SK-BR-3 cell populations infected with either pCMV or pCMV-dn3 were also used for experiments as outlined in the original grant proposal to screen for the levels of HER-2, HER-3, PI 3-kinase and MAP-kinase activation, as well as their tumorigenicity *in vivo*. These cell populations showed no dramatic differences in the levels of HER-2, HER-3, PI 3-kinase and MAP-kinase activation, and the BT-474-derived cells showed no significant reduction in tumor growth in scid mice *in vivo* (data not shown). However, these results were difficult to interpret because this may also indicate that the mass selected and extensively passaged cell populations may not retain many cells expressing very high levels of dominant negative HER-3 if they are being selected out during passaging in culture.

In order to further investigate the expression of dominant negative HER-3 in cell colonies immediately after selection on G418, additional anti-HER-3 immunocytochemistry was performed (Fig. 7). While many of the smaller and dying colonies infected with pCMV-dn3 expressed very high levels of dominant negative HER-3 (Fig. 7B), other colonies showed only moderate or lower levels of dominant negative HER-3 expression (Fig 7C). Some heterogeneity of staining was also seen within individual colonies. A number of the slow growing cell clones from pCMV-dn3-infected BT-474 cell populations were also isolated and screened for their expression of dominant negative HER-3. Additionally, the acquisition of a polyclonal anti-

HER-3 antiserum directed against the extracellular domain of HER-3 (Transduction Labs) has now allowed us to directly identify the dominant negative HER-3 protein in Western blots for these clones at early passage in culture (Fig. 8). Interestingly, the slowest growing clones which required a much longer time to grow out were the samples that showed the highest levels of the approximately 100 kD dominant negative HER-3 protein (Fig. 8, Lanes 2, 6, 8 and 9). In addition, the dominant negative form of HER-3 was seen as a broad band in Westerns, possibly indicative of differential glycosylation and/or other modification of the ectopic protein in genetically engineered cell lines. There may be some potential for using these BT-474-derived cell clones at early passage. However, we may have difficulty in maintaining cell lines with stable high-level dominant negative HER-3 gene expression during extensive passaging in culture because these cells may require constitutive HER-2/HER-3 function for propagation in culture. In addition, preliminary analysis of later passages of these cell clones did not appear to show as high a level of the ectopic dominant negative HER-3 in Western blots (data not shown). This further supports the contention that we may be progressively selecting against the expression of the ectopic gene in these cells after extensive passaging in culture.

If the highest level expressing cells are being selected out because of the inhibitory effects of the ectopic gene, this may necessitate the use of an inducible expression vector to directly study the full extent of the effects of dominant negative HER-3 in those cell lines requiring HER-2/HER-3 constitutive function for propagation in culture. Therefore, we have now developed retroviral expression vectors for engineering cell lines that express regulatable dominant negative HER-3 using the Tetracycline repressor ("Tet-Off") system. The tetracycline-repressible pRevTRE retroviral expression vector facilitates the regulated expression of ectopic genes in mammalian cells (Clontech). Therefore, we constructed an inducible dominant negative HER-3 vector using the pRevTRE vector as previously proposed. pRevTRE contains Sal I and Cla I sites that were used to insert the Sal I-Cla I dominant negative HER-3 fragment isolated from pBK-dn3 (45) into pRevTRE to generate pRevTRE-dn3 (Fig. 9B). We also constructed a control vector, pRevTRE-Lac Z, which was used to determine the levels of tetracycline repressor activity in cell lines stably infected with pRevTet-Off (which contains the tetracycline repressor and neomycin-resistance genes). pRevTRE-Lac Z was cloned by inserting the Hind III-Nar I Lac Z fragment isolated from pSV- β -Galactosidase (Promega) into the Hind III and Cla I sites (Nar I ends are also compatible with Cla I ends) in pRevTRE to generate pRevTRE-Lac Z (Fig. 9A). The proper construction of the vectors was verified with extensive restriction digest analysis (Fig. 10). The newly constructed pRevTRE-dn3 vector was then used to infect cells that had been previously infected with pRevTet-Off (using media from PA-17 cells transfected with pRevTet-Off and selected on G418), selected on G418 and screened for tetracycline repressor function. A number of cell clones infected with pRevTet-Off were isolated and then screened using the pRevTRE-Lac Z vector for transfection and β -galactosidase assays to assess the tetracycline repressor function in different BT-474pRevTet-Off cell clones infected with pRevTet-Off (Fig. 11).

The pRevTRE vector utilizes a hygromycin resistance gene, and selection of cells infected with pRevTRE-dn-3 on hygromycin allowed us to derive cell lines that express the tetracycline repressor protein as well as the dominant negative HER-3 gene driven by the CMV-TRE fusion promoter. PA-317 packaging cells were transfected with pRevTRE or pRevTRE-dn3, selected on hygromycin and these cell lines were then used to infect the tetracycline repressor-positive BT-474pRevTet-Off C3 cell clone with pRevTRE or pRevTRE-dn3. The infected cells were all cultured continuously in the presence of 10 μ g/ml tetracycline to keep the

dominant negative HER-3 gene off during the selection on hygromycin, cloning and passaging of cell lines prior to experimentation. We isolated a number of different BT-474pRevTRE-dn3 cell clones that were then passaged for further use in the presence of tetracycline. BT-474pRevTet-Off C3 cells infected with pRevTRE and selected on hygromycin were also used as a control. Prior to the start of experiments (at least 10 days), tetracycline was removed from one set of the cells, while the others were continuously cultured with tetracycline. We then screened a number of the BT-474pRevTRE-dn3 cell clones for the expression of dominant negative HER-3 as well as the level of tyrosine-phosphorylated HER-2/HER-3. Immunocytochemistry was used to screen cell clones for the expression of HER-3 both in the presence and absence of tetracycline. Western blot analysis was also used to measure the levels of dominant negative HER-3 and tyrosine-phosphorylated HER-2/HER-3 in the different cell clones. We have now isolated BT-474pRevTRE-dn3 cell clones that show the induction of dominant negative HER-3. For example, the BT-474pRevTRE-dn3 C49 cell clone showed a high level of dominant negative HER-3 induction as well as a concomitant reduction in tyrosine-phosphorylated HER-2/HER-3 in the absence of tetracycline (Fig. 12). These calls are now being used to measure the effects of inducible dominant negative HER-3 both in culture and *in vivo*.

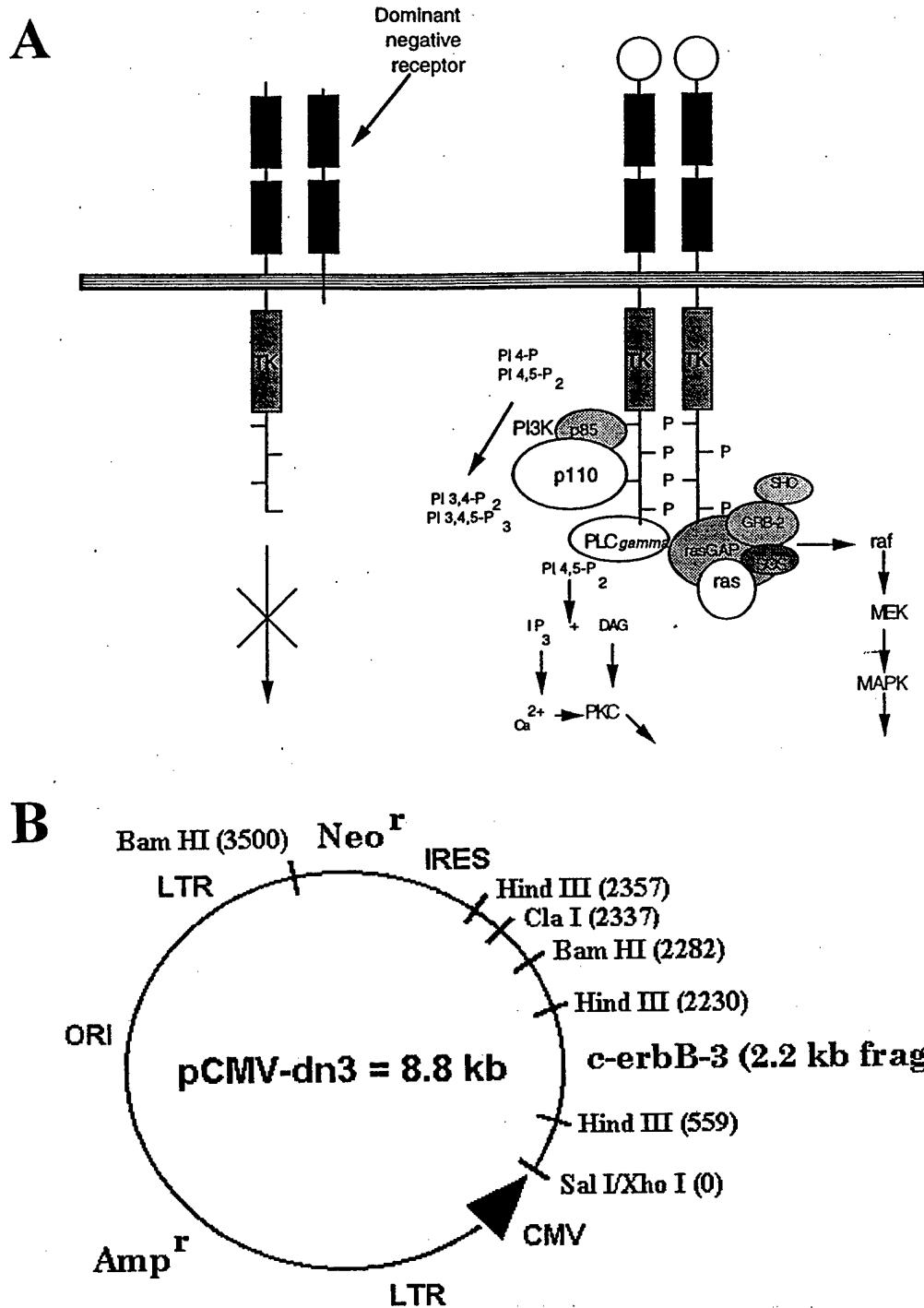


Fig. 1. *Bicistronic Retroviral Expression Vector for Dominant Negative HER-3.* (A) Diagram showing the strategy for blocking wild-type receptor tyrosine kinases with dominant negative (i.e. truncated) receptors. (B) The pCMV-dn3 bicistronic retroviral expression vector was constructed containing a dominant negative form of the HER-3 gene in which most of the cytoplasmic domain of HER-3 was removed. In addition, this vector contains an internal ribosome-binding site (IRES) between the HER-3 fragment and the neo^r gene located downstream, which together, form a single transcription unit when expressed in mammalian cells. The expression of ectopic genes in a bicistronic transcript then allows for the coordinate co-expression of the gene with antibiotic resistance in infected cells selected on antibiotic (45).

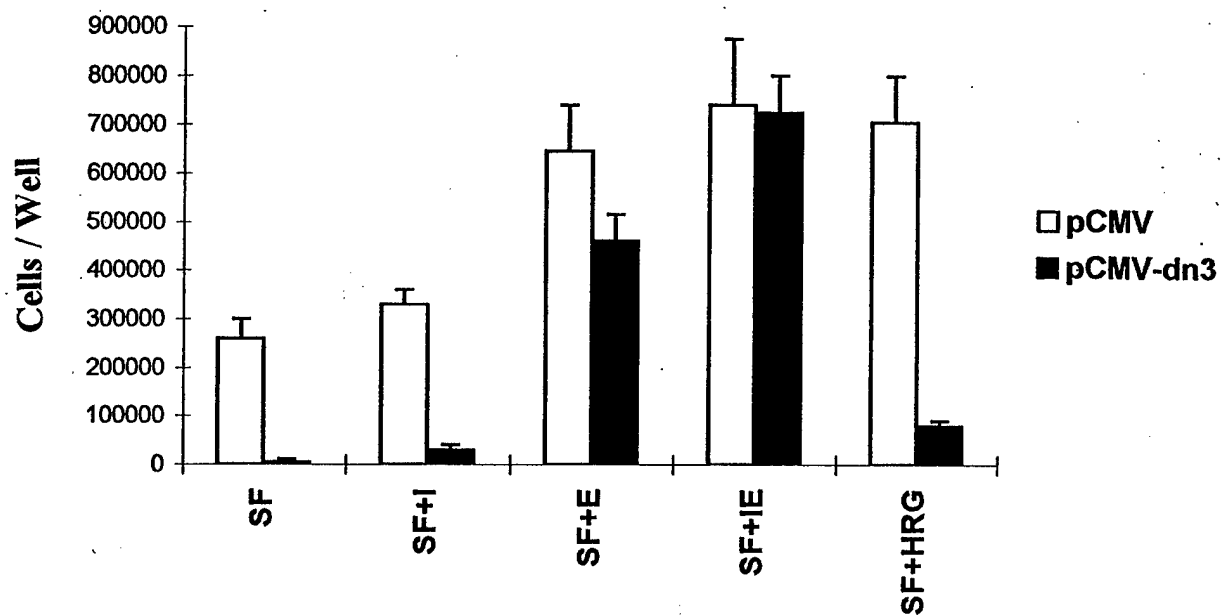


Fig. 2. Dominant negative HER-3 preferentially inhibits the autonomous or HRG-induced proliferation of 21MT-1 cells in culture. Monolayer growth assay showing the proliferation of 21MT-1 cells that had been infected with pCMV or pCMV-dn3 and selected on G418 prior to culturing the cells for 9 days with serum-free (SF) medium, plus 5 ug/ml insulin (I) and/or 10 ng/ml EGF (E), or plus 10 ng/ml HRG- β (HRG). The mean average and standard deviation for triplicate wells is shown.

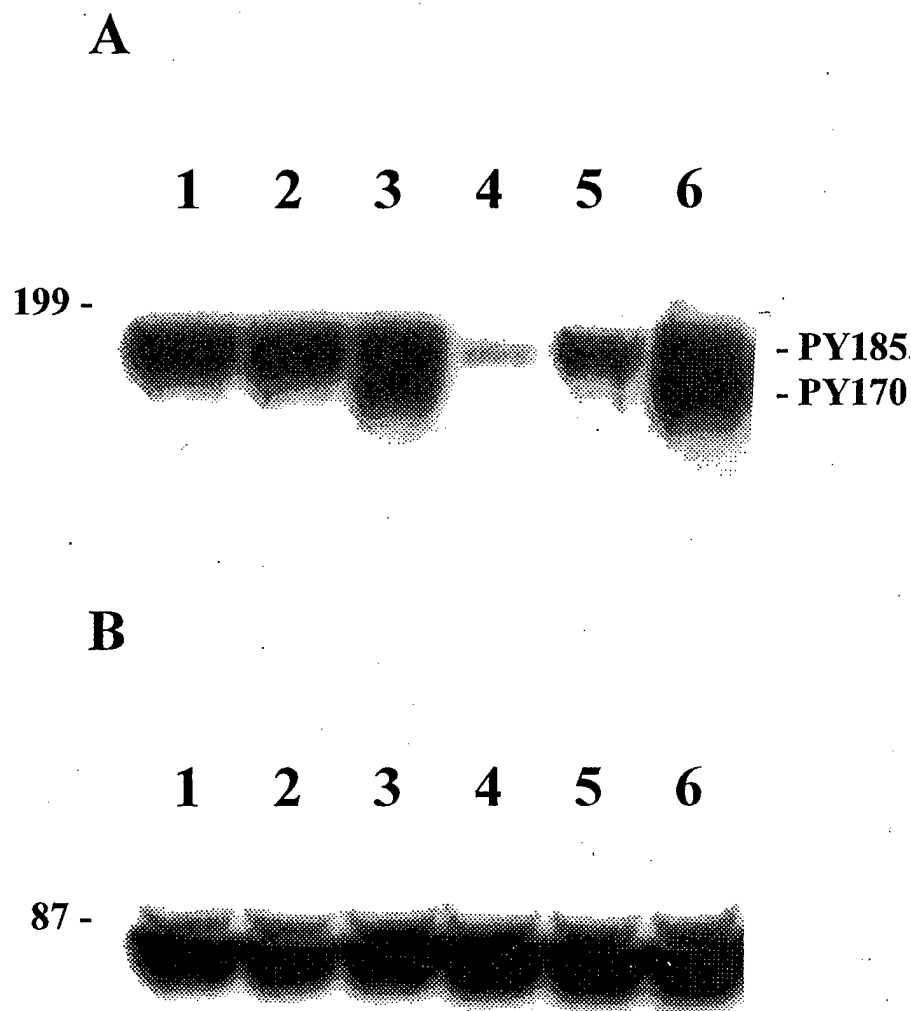


Fig. 3. *Preferential inhibition of HER-2/HER-3 tyrosine phosphorylation in 21MT-1 cells expressing dominant negative HER-3.* A) Samples containing 200 μ g cell lysate protein per lane were immunoblotted with anti-phosphotyrosine antibody. B) The same blot was then reprobbed with anti-p85 antibody as a control to confirm equal loading of the gel. 21MT-1 cells previously infected with either pCMV (Lanes 1-3) or pCMV-dn3 (Lanes 4-6) and selected on G418 were then deprived of growth factors for 48 hours in serum-free, growth factor-free medium and then directly extracted (Lanes 1 and 4), or stimulated with 10 ng/ml HRG- β (Lanes 2 and 5), or stimulated with 10 ng/ml EGF (Lanes 3 and 6) for 10 minutes at 37° C before cell lysate extraction.

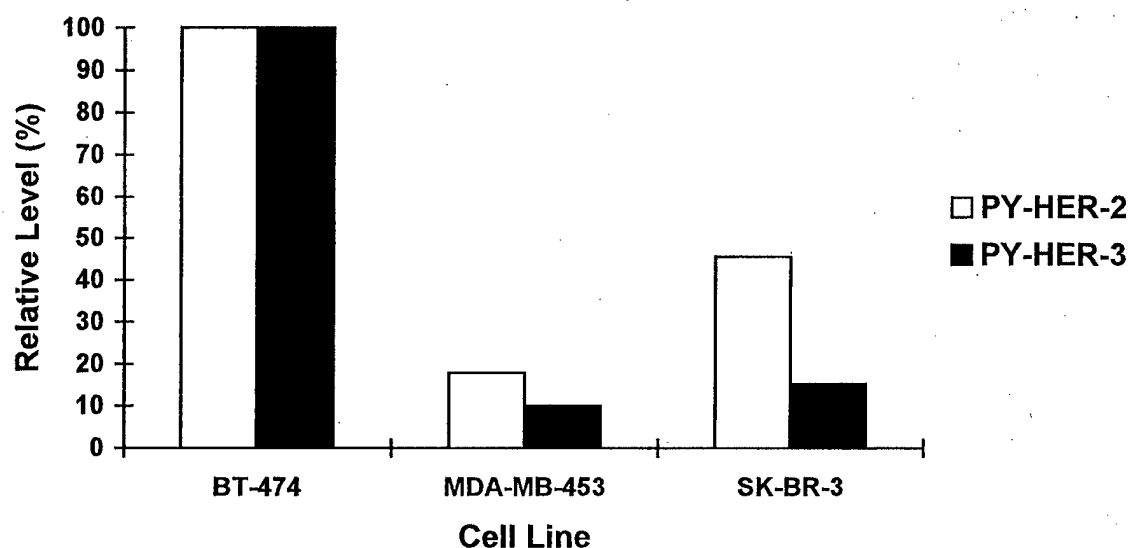


Fig. 4. *Endogenous constitutive activation of HER-2 and HER-3 in BT-474, MDA-MB-453 and SK-BR-3 cells.* Cell lysates from cells incubated for 48 hours without serum or growth factors were immunoprecipitated with anti-phosphotyrosine antibody followed by immunoblotting with anti-HER-2 antibody to measure tyrosine-phosphorylated HER-2, or immunoprecipitated with anti-HER-3 antibody followed by immunoblotting with anti-phosphotyrosine antibody to measure tyrosine-phosphorylated HER-3. Densitometry analysis of film exposed by chemiluminescent blots are shown relative to the highest levels of tyrosine-phosphorylated HER-2 and HER-3 measured in the BT-474 cells.

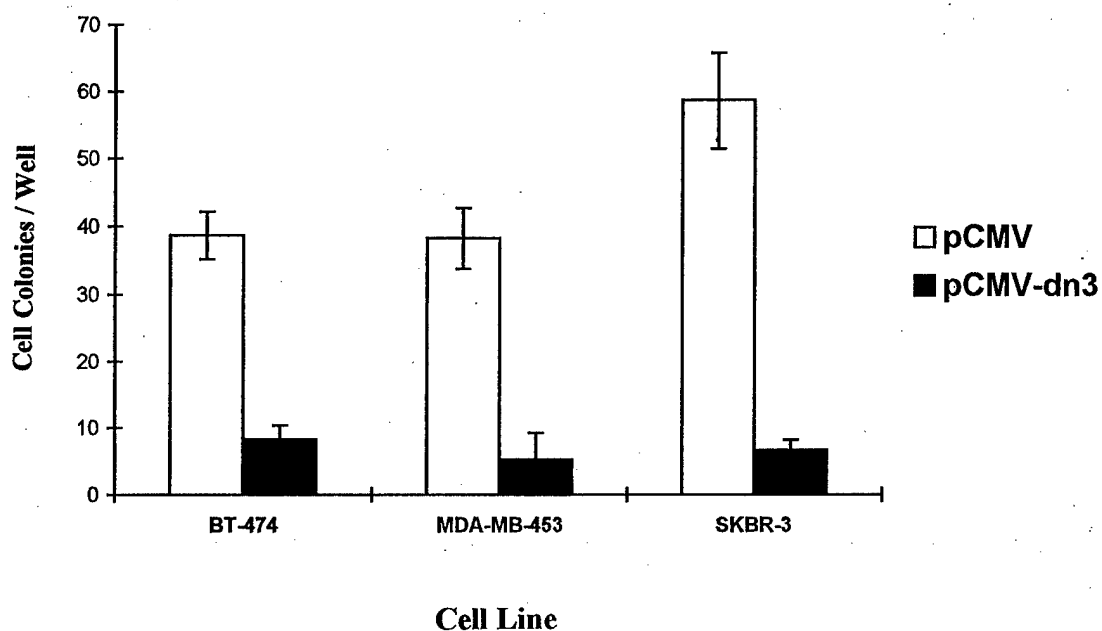


Fig. 5. *Clonal outgrowth of BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV or pCMV-dn3.* BT-474, MDA-MB-453 and SK-BR-3 cells were infected for 3 days with conditioned medium collected from PA-317 packaging cells stably transfected with pCMV or pCMV-dn3. The infected cells were then incubated with fresh medium for 2 days and selected on G418 for a month before counting colonies in 6 well plates. The mean average and standard deviation for triplicate wells is shown.

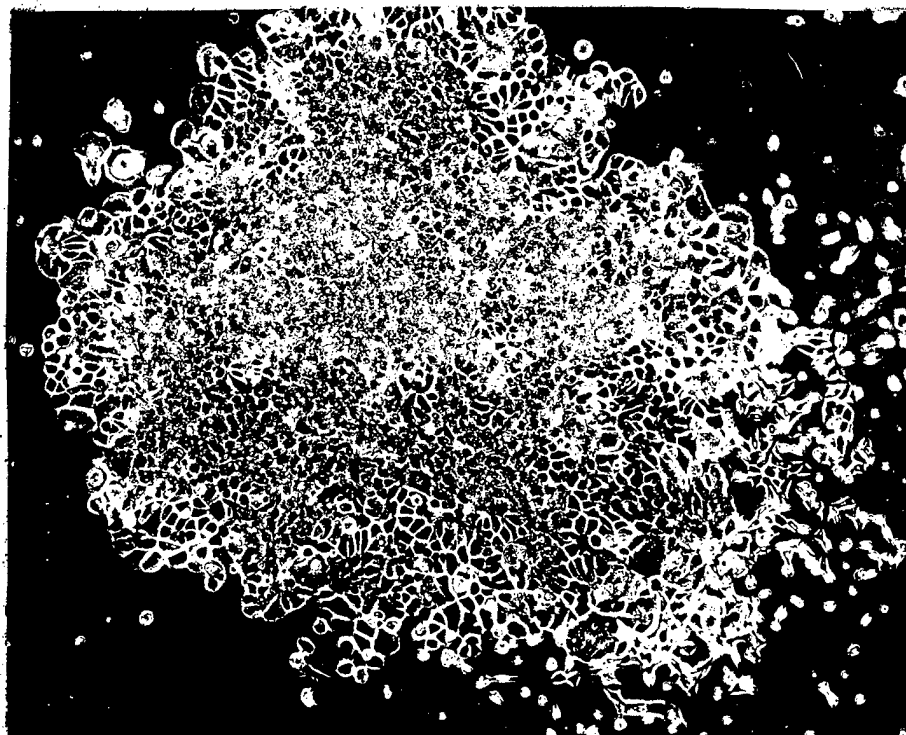
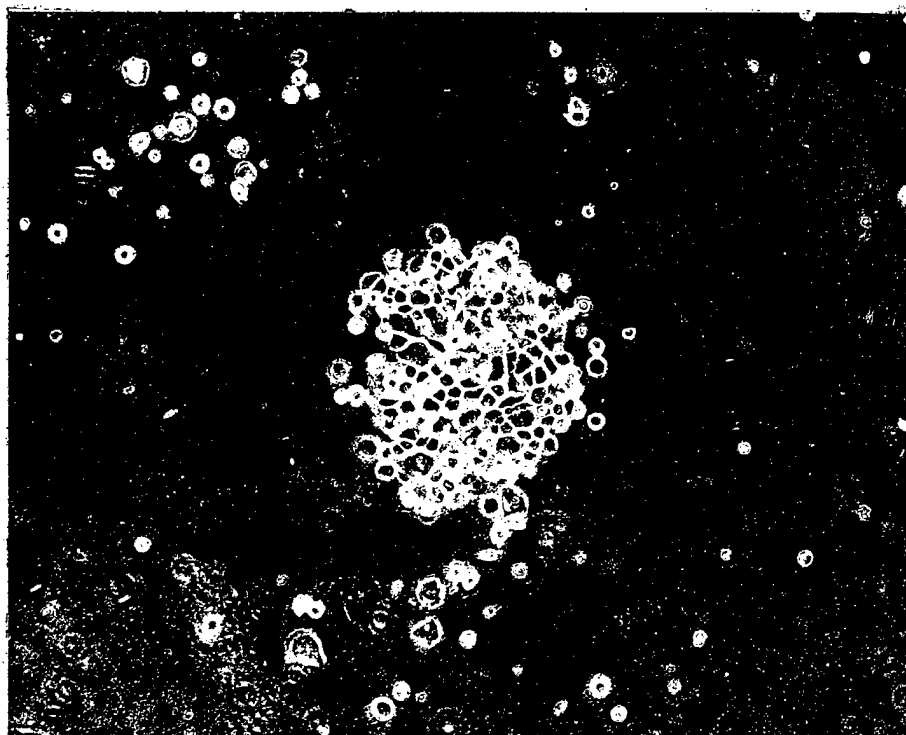
A**B**

Fig. 6. *Morphology of BT-474 cell colonies infected with pCMV or pCMV-dn3.* Phase contrast microscopy of BT-474 cells infected with either pCMV (A) or pCMV-dn3 (B) and selected on G418 for a month in culture.

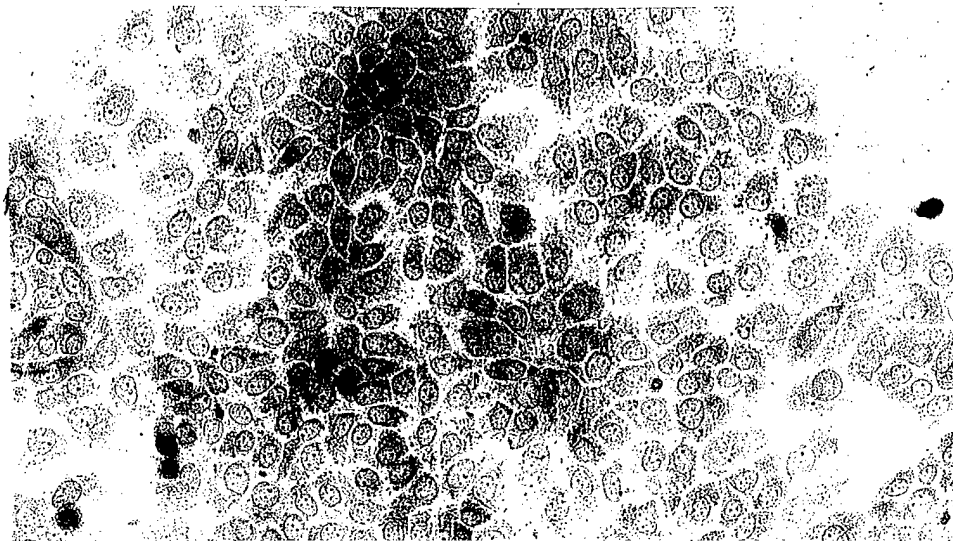
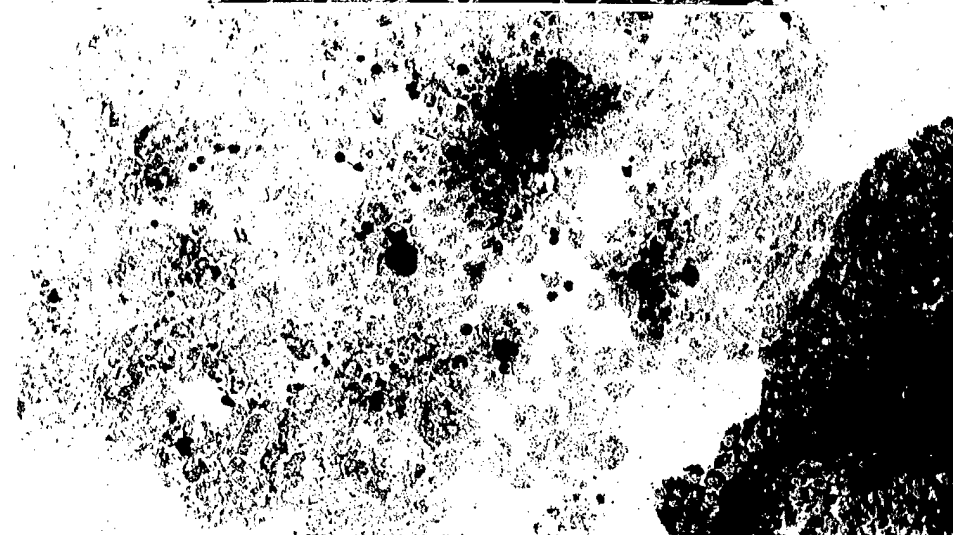
A**B****C**

Fig. 7. *Expression of dominant negative HER-3 in BT-474 cells infected with pCMV-dn3.* Bright field microscopy of anti-HER-3 immunocytochemistry in BT-474 cells infected with either pCMV (A) or pCMV-dn3 (B and C). High levels of ectopic HER-3 was seen in smaller slow growing and dying (not shown) cell colonies. Also notice the heterogeneity in staining between different BT-474 cell colonies infected with pCMV-dn3 (C), where some colonies express low levels (C, upper left) and others express high levels (C, lower right) dominant negative HER-3.

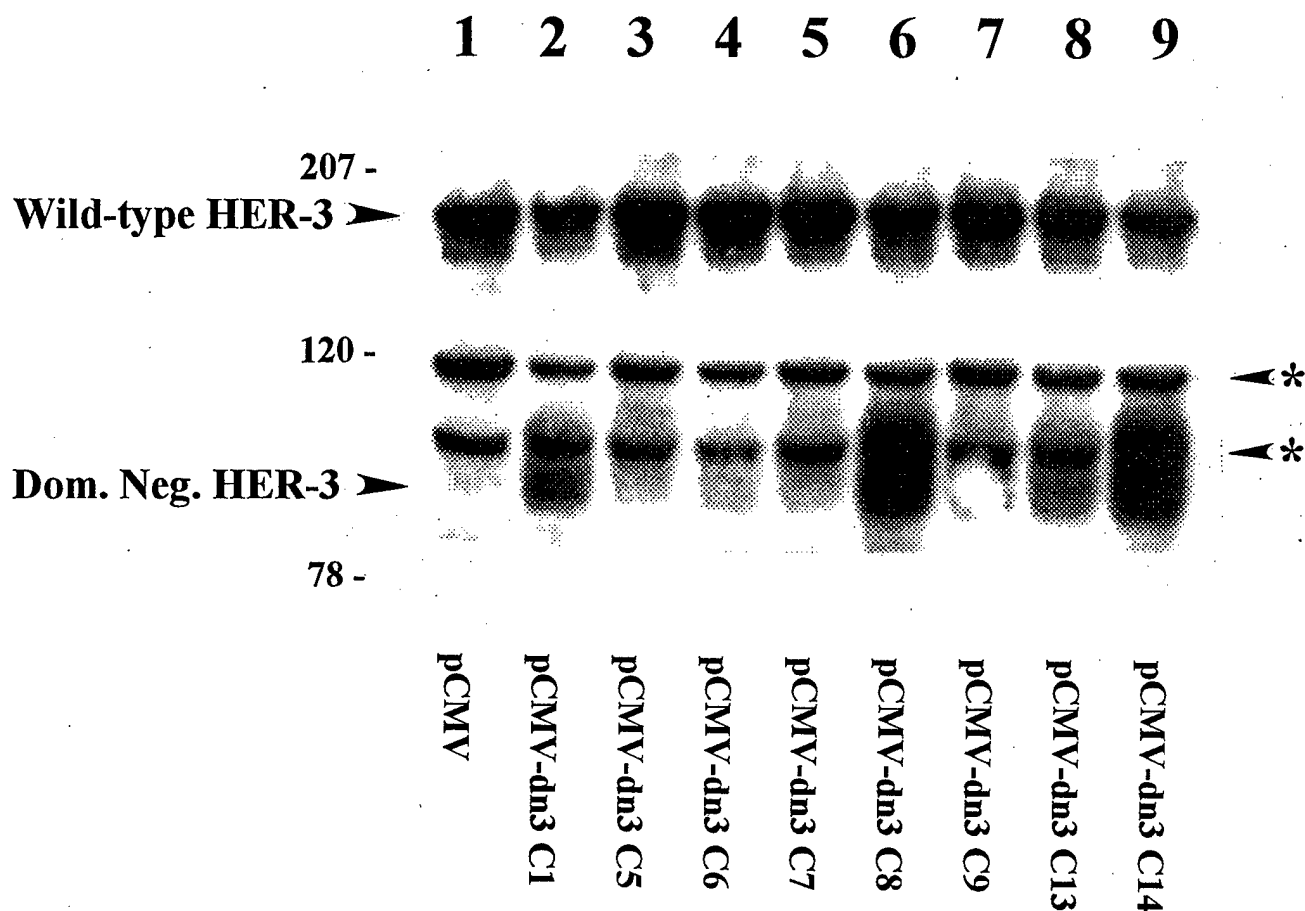
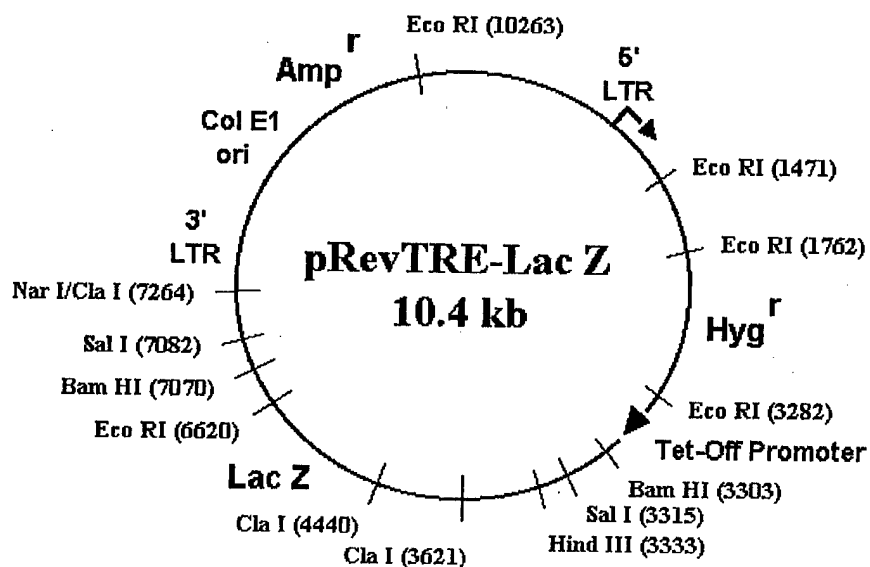


Fig. 8. *Expression of dominant negative HER-3 in BT-474 cell clones infected with pCMV-dn3.* Western blot analysis of HER-3 expression in BT-474 cells infected with pCMV (Lane 1) or different BT-474 cell clones infected with pCMV-dn3 (Lanes 2 -9). Samples containing 100 ug cell lysate protein were immunoblotted with a polyclonal anti-HER-3 antibody raised to the extracellular region of HER-3 to directly identify the approximately 100 kD dominant negative HER-3 protein in cells infected with pCMV-dn3. The clones shown in Lanes 2, 6, 8 and 9 expressed the highest levels of dominant negative HER-3. High molecular weight markers are shown (kD) on the left and non-specific bands are marked (*) on the right.

A



B

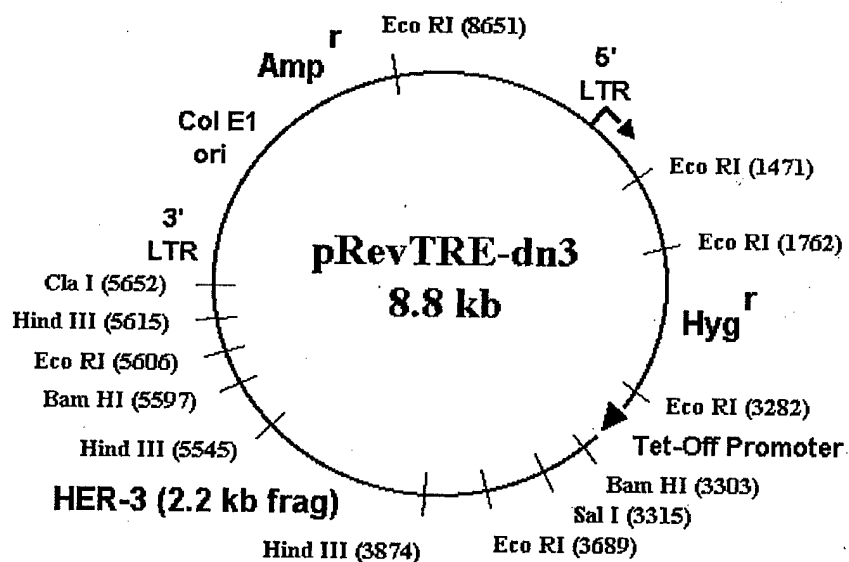


Fig. 9. Tetracycline-repressible vectors for the expression of β -Galactosidase or dominant negative HER-3. The pRevTRE tetracycline-repressible retroviral expression vector (Clontech) was used to construct pRevTRE-Lac Z (A) and pRevTRE-dn3 (B). pRevTRE-Lac Z was used to transfect cell clones previously infected with pRevTet-Off (which contains the tetracycline repressor gene) and screen for tetracycline repressor activity. pRevTRE-dn3 was then introduced into tetracycline repressor-positive cell clones which were then cultured in the presence of tetracycline to keep the ectopic gene off during selection on hygromycin.

Predicted Fragment Length (kb)			
Lane	Enzyme	pRevTRE-Lac Z	pRevTRE-dn3
1)	Bam HI	6.6, 3.8	6.5, 2.3
2)	Cla I	9.6, 0.8	8.8
3)	Eco RI	3.6, 3.3, 1.6, 1.5...	3.1, 1.9, 1.6, 1.5...
4)	Hind III	10.4	7.1, 1.7...
5)	Sal I	6.6, 3.8	8.8

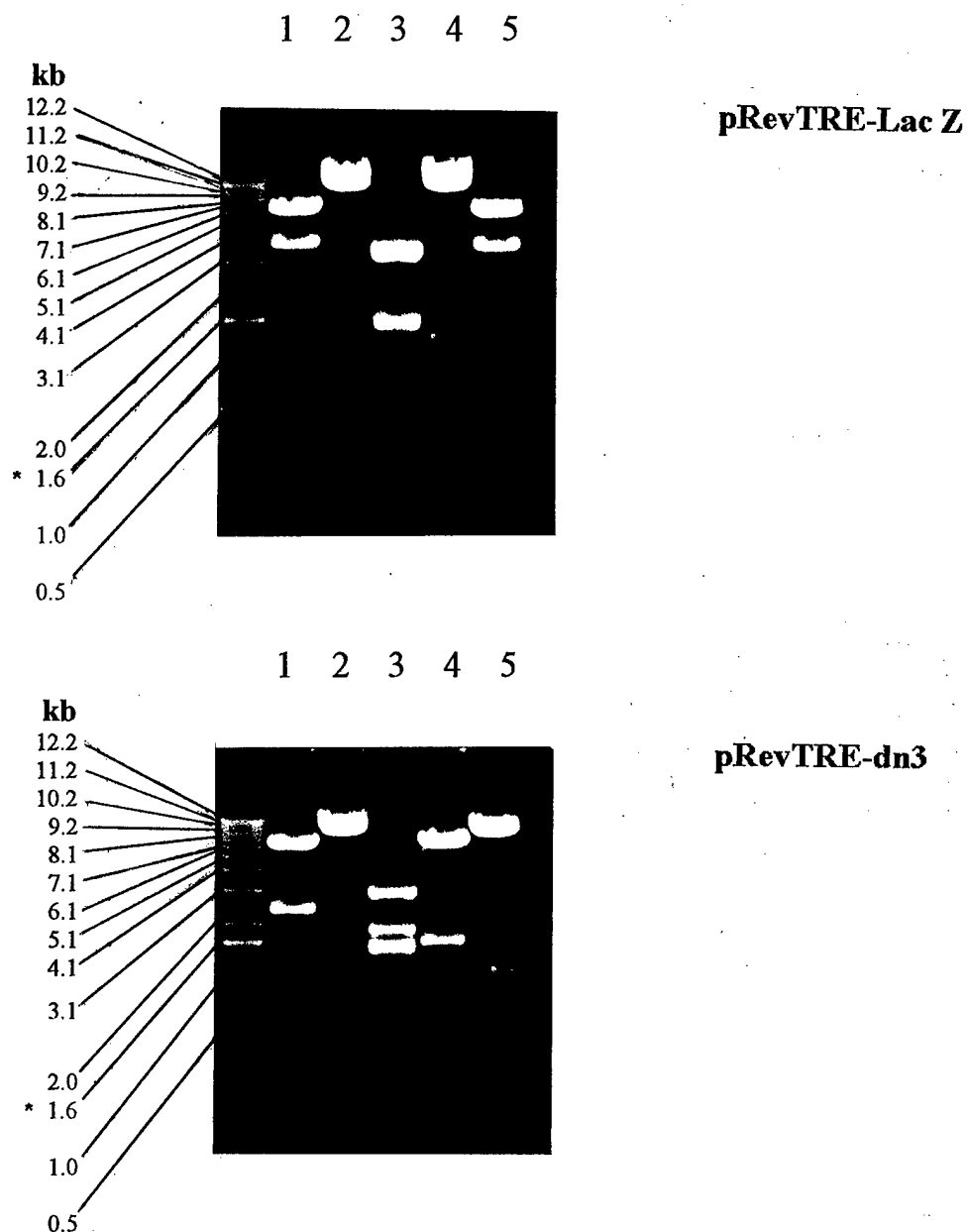


Fig. 10. Restriction digest analysis of pRevTRE-LacZ and pRevTRE-dn3 retroviral expression vectors. Restriction digests of plasmid DNA were electrophoresed in 0.8% agarose gels containing 0.5% ethidium bromide and photographed using UV light. A 1 kb DNA ladder is shown and the 1.6 kb marker (*) is the brightest band in the ladder.

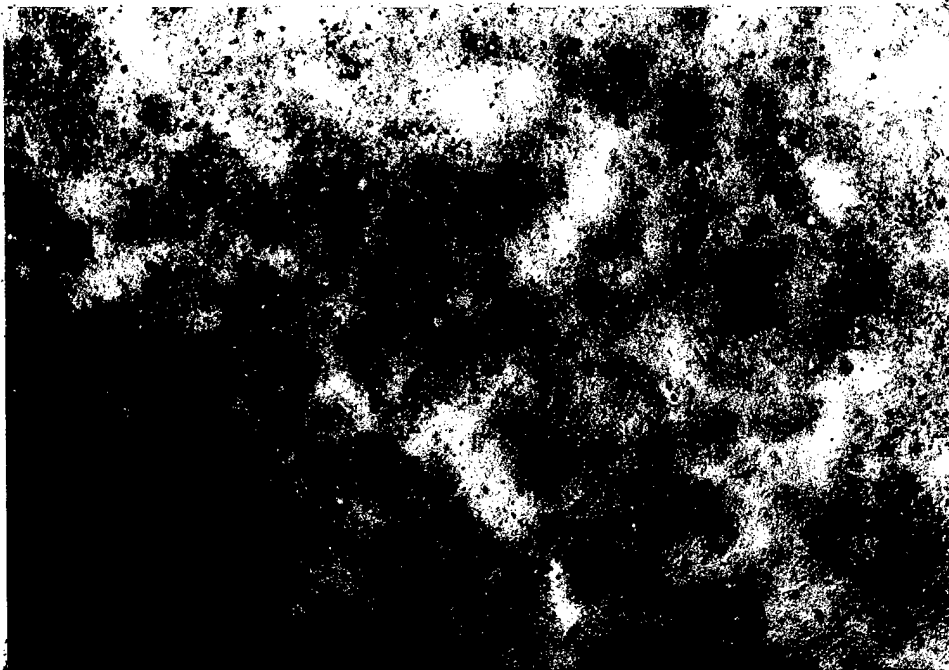
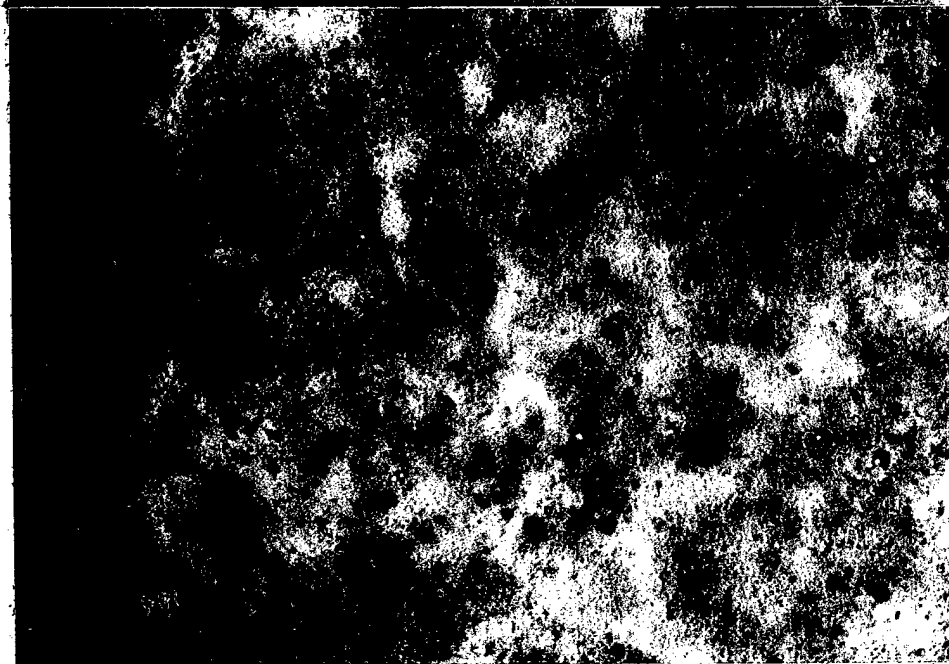
A**B**

Fig. 11. *β -galactosidase activity in BT-474pRevTet-Off cells with or without tetracycline.* In order to screen for tetracycline repressor activity in different BT-474pRevTet-Off cell clones, BT-474pRevTet-Off cells were transiently transfected with pRevTRE-Lac Z and cultured either with (A) or without (B) tetracycline prior to staining for β -galactosidase activity. Bright field microscopy of BT-474pRevTet-Off C3 cells stained for β -Galactosidase activity showed tetracycline repressor function when the marker gene was not expressed in the presence of tetracycline. This cell clone was then used for further infection with pRevTRE-dn3 in order to generate BT-474 cells that express a regulatable dominant negative HER-3 gene.

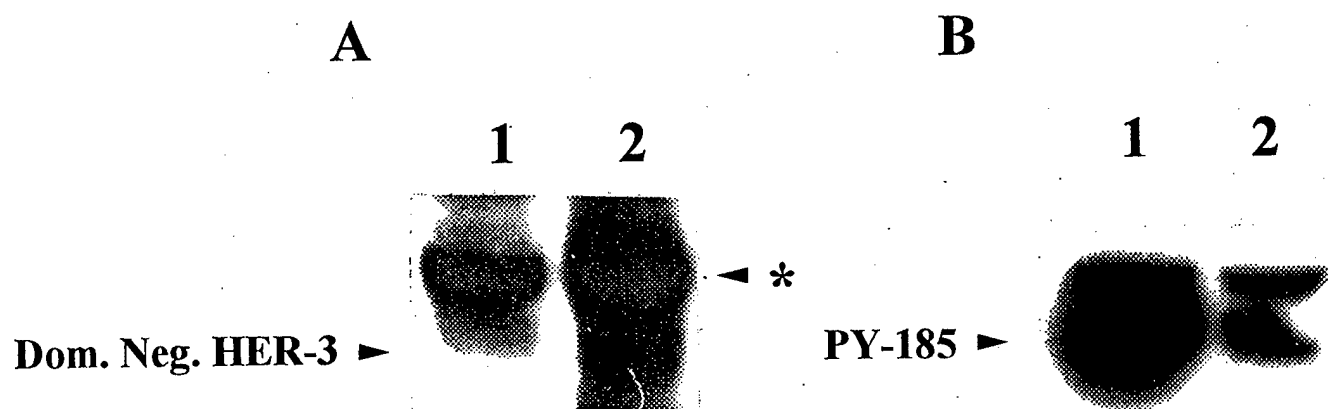


Fig. 12. *Expression of dominant negative HER-3 and tyrosine phosphorylation of HER-2/HER-3 in BT-474pRevTRE-dn3 C49 cells.* A) Samples containing 100 ug cell lysate protein per lane were immunoblotted with the polyclonal anti-HER-3 antibody for the BT-474pRevTRE-dn3 C49 cells with (Lane 1) and without (Lane 2) 10 ug/ml tetracycline. The tetracycline was removed 2 weeks prior to protein extraction and the serum was also removed 48 hours prior to protein extraction. The polyclonal anti-HER-3 antibody raised to the extracellular region of HER-3 was used to identify the approximately 100 kD dominant negative HER-3 protein which is seen in the same region as a non-specific band that is marked (*) on the right. B) Duplicate samples were also immunoblotted with the anti-phosphotyrosine monoclonal antibody for the BT-474pRevTRE-dn3 C49 cells with (Lane 1) and without (Lane 2) 10 ug/ml tetracycline to measure the relative levels of tyrosine-phosphorylated HER-2/HER-3.

KEY RESEARCH ACCOMPLISHMENTS

- The 21MT-1-derived cells were used for additional studies of the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*.
- BT-474, MDA-MB-453 and SK-BR-3 cells were infected with pCMV and pCMV-dn3 and a number of cell clones infected with pCMV-dn3 were also isolated for further characterization.
- BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV and pCMV-dn3 were characterized for the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses *in vivo*.
- Additional vectors and cell lines were developed to study the effects of regulatable dominant negative HER-3 gene expression in BT-474 cells using the "Tet-Off" promoter system.

REPORTABLE OUTCOMES

A manuscript describing most of the work done with the H16N-2 and 21MT-1 cell lines was published earlier (45). Another manuscript containing additional data and work using the BT-474, MDA-MB-453 and SK-BR-3 cell lines is presently in preparation. A poster describing this work was also presented at the 2002 Department of Defense "Era of Hope" meeting.

CONCLUSIONS

Amplification and overexpression of the HER-2 gene is involved in the oncogenic transformation of mammary epithelial cells in approximately a third of breast cancer patients. In those individuals with HER-2 gene amplification, this dominant genetic event is likely to be the principle change which drives malignancy because HER-2 is such a potent oncogene when highly overexpressed in experimental systems. However, our understanding is still fragmentary concerning the exact mechanisms by which signals for cell growth are constitutively activated in breast cancer cells with HER-2 gene amplification, and methods for permanently inhibiting the constitutive activation of signaling in such cells have not yet met with great success. Our previous work showed that the high-level overexpression of HER-2 in 21MT-1 cells was associated with the constitutive activation of HER-2/HER-3, PI 3-kinase, and growth factor independence in culture (22). By constitutively activating key mitogenic signaling pathways to a level that is effective for autonomous growth, tumor cells escape the normal controls on cell cycle regulation. Therefore, we sought to experimentally assess the importance of the cooperative interactions between HER-2 and HER-3 during the growth factor-independent proliferation of breast cancer cells with HER-2 gene amplification, as well as in cells stimulated by exogenous HRG. Our data now confirm the importance of the HER-2/HER-3 heterodimer interaction for receptor activation and the recruiting of key signaling molecules in 21MT-1 breast cancer cells with HER-2 gene amplification, as well as in normal H16N-2 cells stimulated by HRG (45). Dominant negative HER-3 also potently inhibited the growth factor-independent and anchorage-independent growth of the 21MT-1 cells in culture (45). Therefore, our work studying the interaction between HER-2 and HER-3 offers exciting new opportunities for blocking the mechanism of autonomous growth in breast cancer cells with HER-2 gene amplification.

By itself, HER-3 is known to be almost completely kinase deficient (as would be expected from sequence analysis showing deleterious substitutions in the enzymatic site) and is not able to activate signaling in-and-of-itself in genetically engineered cell lines that do not co-express any of the other HER kinases. However, while the other HERs have active kinase domains, HER-3 contains multiple additional docking sites for PI 3-kinase and SHC proteins not found in the other HERs. HER-2 is also known to be an especially active tyrosine kinase that exhibits ligand-independent activation when overexpressed. These combined considerations (i.e. HER-3 docking sites combined with HER-2 kinase potential) may account for the especially potent activation of signaling induced by HER-2/HER-3 heterodimers in response to HRG in H16N-2 cells (22, 45, 46), and that is constitutively activated in 21MT-1 cells (22, 45). Interestingly, the blocking of HER-2/HER-3 function with dominant negative HER-3 showed specificity in that the H16N-2 and 21MT-1 cells infected with pCMV-dn3 and selected on G418 still proliferated in response to exogenous EGF. This suggested that HER-1/HER-3 is not as effectively inhibited by dominant negative HER-3 as is HER-2/HER-3 at a given level of dominant negative HER-3 expression or that the interaction between EGFR and HER-3 is not required for EGF-induced proliferation. As mentioned above, HER-1 and HER-3 interact to some extent in these (46) and other cell lines (34, 35), and this interaction may be required for EGF-stimulated growth (46). However, the relative affinity of HER-1/HER-3 heterodimers is very weak compared to HER-2/HER-3 heterodimers when compared with cross-linking analysis (42-44). Therefore, in order to further investigate the preferential effect that dominant negative HER-3 had on HER-2/HER-3-mediated growth, we also compared the effects of dominant

negative HER-3 on both HRG- and EGF-induced activation of HERs with anti-phosphotyrosine immunoblotting. While dominant negative HER-3 inhibited the levels of tyrosine phosphorylated HER-2/HER-3 in 21MT-1 cells, the EGF-induced tyrosine phosphorylation of EGFR-containing dimers was not apparently affected by dominant negative HER-3. This result was also consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers at a given level of dominant negative HER-3 in genetically engineered cell lines.

It is not entirely clear why so many breast carcinoma cell lines are not tumorigenic in immunodeficient mice, but apparently this is a common problem (47), and most previous reports studying HER-2-mediated cell transformation have employed fibroblasts genetically engineered to overexpress HER-2 for their *in vivo* studies rather than spontaneously derived breast carcinoma cell lines. So far, we have only found the BT-474 cells to be tumorigenic in immunodeficient mice, so we have focused most of our attention on this cell line for future experiments. We generated BT-474, MDA-MB-453 and SK-BR-3 cells stably infected with either pCMV or pCMV-dn3, and have screened cells infected with pCMV-dn3 for the expression of dominant negative HER-3. We used these and other newly developed BT-474-derived cell clones to further study the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses as originally outlined in the grant proposal. While we have generated BT-474, MDA-MB-453 and SK-BR-3 cells infected with these vectors, we have also seen a much lower number of colonies growing out during selection on G418 for cells infected with the pCMV-dn3 than for those infected with pCMV. Furthermore, we noticed that many of the colonies that started to grow out in plates infected with pCMV-dn3 did not continue to grow, suggesting that the proliferation and survival of many of the cell clones infected with pCMV-dn3 was inhibited by the expression of dominant negative HER-3. This is also consistent with the heterogeneity in anti-HER-3 staining seen for mass selected and extensively passaged cells. In addition, we have now found that cells infected with pCMV-dn3 and stained immediately after being selected on G418 showed high-level expression of dominant negative HER-3 in smaller slow growing or dying colonies. Therefore, the low number of colonies seen for cells infected with pCMV-dn3 indicates that the growth of many of the cells expressing the highest levels of dominant negative HER-3 may be inhibited and thus only form small or attenuated colonies, while clones expressing lower levels of dominant negative HER-3 may be selected for during subsequent passaging in culture.

Little is known about the growth factor requirements for BT-474, MDA-MB-453, and SK-BR-3 cells, which are all cultured under undefined conditions in medium containing 10% fetal calf serum. Therefore, it may be difficult to maintain cell populations that express high levels of dominant negative HER-3 under conditions where constitutive and regulative growth responses have not yet been determined. Due to stoichiometric considerations, it is crucial to generate cells that express very high levels of the dominant negative HER-3 in order to effectively block wild-type HER-2/HER-3 function. As mentioned above, if the highest level expressing cells are being selected out because of the inhibitory effects of dominant negative HER-3, this may necessitate the use of a regulatable expression system to directly study the full extent of the effects of dominant negative HER-3 in these cell lines. The tetracycline-repressible pRevTRE retroviral expression vector facilitates the inhibition of ectopic genes in mammalian cells during selection on antibiotic (Clontech). Therefore, we constructed a tetracycline-repressible dominant negative HER-3 vector for further use in these cell lines. The newly constructed vector was then used to infect BT-474 cells that had been infected with pRevTet-Off,

selected on G418 and screened for functional tetracycline repressor activity by transfection of the pRev-TRE-Lac Z vector and culture with or without tetracycline prior to assessing the level of β -galactosidase activity. The pRevTRE vector utilizes a hygromycin resistance gene and double selection on G418 and hygromycin then allowed us to derive cell lines that express the tetracycline transcriptional regulator protein and also contain the dominant negative HER-3 gene driven by the CMV-TRE fusion promoter that is repressed by the tetracycline repressor. Cells infected with the regulatable form of dominant negative HER-3 were then cultured in the presence of tetracycline to keep the dominant negative HER-3 gene off during selection on hygromycin. Cells with (as a control) and without tetracycline are presently being used for experiments to determine the effectiveness of dominant negative HER-3 in inhibiting HER-2/HER-3 activation and growth responses. This "Tet-Off" system allows us to maintain the cells with the ectopic gene off in the presence of tetracycline and, with removal of tetracycline, the cells turn on the dominant negative HER-3 gene just prior to the start of the experiment. This system apparently also allows for a wide range of repression through the TRE element, depending on the concentration of tetracycline employed (Clontech). This will also be useful for directly studying the stoichiometric aspect of receptor inhibition in experiments pertaining to future grant proposals.

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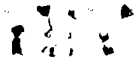
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APPENDICES

None.